Molecular Characterization of Quinine/Quinidine Drug-Dependent Antibody Platelet Interaction Using Monoclonal Antibodies

By Michael C. Berndt, Beng H. Chong, Helen A. Bull, Heddy Zola, and Peter A. Castaldi

Two murine monoclonal antibodies, FMC 25 and AN 51, directed against distinct epitopes on the glycoprotein lb complex, have been used to further define the mechanism of quinine/quinidine drug-dependent antibody interaction with platelets. FMC 25, directed against an epitope on glycoprotein IX, had no effect on platelet aggregation induced by collagen or adenosine diphosphate and little, if any, effect on ristocetin-induced platelet agglutination. FMC 25 and its (Fab)_2 fragment, however, were potent inhibitors of drug-dependent antibody-induced platelet aggregation and blocked binding of drug-dependent antibody to platelets as assessed by indirect platelet immunofluorescence. In contrast, AN 51, directed against an epitope on the a-subunit of glycoprotein lb, blocked ristocetin-induced, factor VIII/von Willebrand factor (FVIII/vWF)-dependent platelet agglutination but not drug-dependent antibody-induced platelet aggregation or binding of drug-dependent antibody to platelets. Selective proteolytic removal of the majority of the a-subunit of glycoprotein lb (glycocalcin) from platelets by treatment with calcium-dependent protease did not affect binding of drug-dependent antibody. In addition, a quinine-dependent antiplatelet antibody immunoprecipitated glycoprotein lb complex from normal platelets and the membrane-associated proteolytic remnant of the glycoprotein lb complex from calcium-dependent protease-treated platelets. Preincubation of drug-dependent antibody with purified glycoprotein lb complex inhibited subsequent binding of antibody to platelets, but the separated components, glycoprotein lb and glycoprotein IX, were both ineffective, suggesting that the normal interaction between glycoprotein lb and glycoprotein IX in the intact complex was necessary for drug-dependent antibody recognition. The functional response of platelets to drug-dependent antibody was not mediated by way of platelet Fc receptor, since aggregation of washed platelets by acetone-aggregated IgG was not inhibited by FMC 25 (Fab)_2. FVIII/vWF was not required for drug-dependent antibody-induced platelet aggregation. The combined evidence is consistent with quinine/quinidine-dependent antibody-platelet interaction occurring by way of a FVIII/vWF-independent, Fc receptor-independent mechanism that probably involves binding of antibody to glycoprotein IX or the β-subunit of glycoprotein lb or both.

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MATERIALS AND METHODS

Materials. Bovine serum albumin (fraction V), leupeptin, quinidine sulfate, and quinidine sulfate were purchased from Sigma Chemical Co, St Louis; sodium periodate (Univar), from Ajax Chemicals, Sydney, Australia; Pansorbin and diisopropylfluorophosphate (DFP), from Calbiochem, La Jolla, Calif; Triton X-100, from BDH, Poole, England; pepsin, from Worthington, Freehold, NJ; Iodobeads, from Pierce, Rockford, Ill; DEAE-Affigel Blue and Affigel-10 were from Bio-Rad, Richmond, Calif; affinity-purified goat antimouse IgG (γ), from Kirkegaard and Perry Laboratories, Gaithersburg, Md; fluorescein-labeled rabbit antihuman IgG (monospecific) was obtained from Dako-immunoglobulins (a/s) Copenhagen; NaCl from New England Nuclear, Boston; and 3H-sodium phosphate from New England Nuclear, Boston.
Drug-dependent antibody platelet binding

Drugs-dependent antibodies. These studies used serum from three patients with quinine-dependent antibodies (B, P, and V) and three patients with quinidine-dependent antibodies (D, W, and Y). IgG was purified from two of the patients with quinidine-dependent antibodies (D and W) by performing two 0% to 40% ammonium sulfate fractionations, followed by chromatography on DEAE-Affigel Blue. The purity of IgG was >95% homogenous as assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. AN 51 (as purified IgG), obtained from Dr A.J. McMichael, Nuffield, England, was a gift of Dr K. Bradstock of our department. FMC 25 (Fab2) was prepared from FMC 25 IgG by pepsin digestion as described by Coller et al. On SDS-polyacrylamide gel electrophoresis, under nonreduced conditions, the FMC 25 (Fab2) gave a single band of 100,000 mol wt.

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Purification of the human glycoprotein I complex. The immunoaffinity procedure for purification of the glycoprotein I complex, glycoprotein Ib and glycoprotein IX, was performed in detail elsewhere. Briefly, the platelets from 10 units of 24-hour-old platelets were suspended in 150 mL of Buffer A (2 x 10^10/mL), loaded onto 4 x 30-mL columns of FMC 25-Affigel 10 (3.6 mg of FMC 25 per milliliter of agarose). After thorough washing, the glycoprotein Ib complex was eluted with 0.01 mol/L Tris, 0.001 mol/L EDTA, 0.001 mol/L N-ethylmaleimide, 0.1% (vol/vol) Triton X-100, 0.02% (wt/vol) NaN3, pH 7.4, and then centrifuged at 100,000 g. The supernatant was loaded onto a 10 x 1-cm column of WM 23-Affigel 10 (2.6 mg of WM 23 per milliliter of agarose). After thorough washing, the glycoprotein IX was eluted with 0.1 mol/L glycine, 0.1% (vol/vol) Triton X-100, pH 2.4. The eluted fractions were immediately neutralized by the addition of one fifth final volume of 1 mol/L Tris buffer, 0.1% (vol/vol) Triton X-100, 0.1% (wt/vol) NaN3, pH 8.0. Glycoprotein Ib was then eluted with washing with 0.01 mol/L Tris, 0.13 mol/L NaCl, 0.001 mol/L EDTA, 0.1% (vol/vol) Triton X-100, 0.02% (wt/vol) NaN3, pH 7.4. Triton X-100 was removed from the preparations of glycoprotein Ib complex, glycoprotein Ib and glycoprotein IX, by chromatography on Extract-i-gel D (Pierce), according to the manufacturer’s instructions. The a- and fl-subunits of glycoprotein lb and glycoprotein IX were extracted from gel slices after SDS-polyacrylamide gel electrophoresis (under reducing conditions) according to the method described by McEvoy et al. Glycoprotein Ib complex, glycoprotein iba, ibb, and IX (at ^100 mg/mL) were labeled by iododecatalyzed radioiodination according to the manufacturer’s recommendations (Pierce).

Platelet preparation and labeling. For the preparation of washed platelets, six parts of venous blood were collected into one part of acid-citrate-dextrose (ACD) as anticoagulant. ACD contained 2.5% (wt/vol) trisodium citrate, 2% (wt/vol) b-glucose, and 1.5% (wt/vol) citric acid. The blood was centrifuged at 160 g for 20 minutes and the platelet-rich plasma (PRP) removed. The PRP was centrifuged at 800 g for 15 minutes and the platelets resuspended and washed by centrifugation, as previously described. The washed platelets were suspended at 10^7/mL in EDTA-HEPES-saline (EHS) buffer and the platelet membrane glycoproteins labeled with sodium periodate/H₂-sodium borohydride. EHS buffer contained 0.01 mol/L HEPES, 0.15 mol/L NaCl, 0.001 mol/L EDTA, pH 7.6. For aggregation studies, blood was collected into 3.18% sodium citrate (9 vol blood to 1 vol anticoagulant). PRP was prepared as described above. Platelet-poor plasma (PPP) was prepared by centrifugation of PRP at 1,000 g for 10 minutes. For aggregation studies with washed platelets, the platelets were finally suspended in Tyrode’s solution such that the final platelet concentration in the aggregometer was 3 x 10^6/mL. Tyrode’s solution contained 0.138 mol/L sodium chloride, 0.0029 mol/L potassium chloride, 0.0012 mol/L sodium bicarbonate, 0.0036 mol/L sodium phosphate, 0.0055 mol/L glucose, 0.0005 mol/L magnesium chloride, and 0.0018 mol/L calcium chloride, pH 7.4. For the indirect platelet immunofluorescence, the platelets were washed and fixed with 1% (wt/vol) paraformaldehyde as previously described.

Calcium-dependent protease treatment of washed platelets. Washed platelets in Ca²⁺-Mg²⁺-free Tyrode’s solution were equilibrated with calcium-dependent protease (0.5 U/mL) in the presence of 2 mmol/L calcium at 37 °C for 30 minutes. An aliquot of platelets was checked for ristocetin cofactor activity in the aggregometer using 10% normal plasma and 1 mg/mL of ristocetin. If the aggregation was not completely abolished, the platelets were pelleted by centrifugation at 800 g, resuspended to the same volume in fresh buffer, and the proteolysis repeated. Ristocetin cofactor activity was generally completely abolished after two steps. One unit of calcium-dependent protease activity was defined as the amount of enzyme required to produce an absorbance change of 1 absorbance unit per
hour at 37 °C under the assay conditions (0.1 mol/L Tris, 0.24% [wt/vol] casein, 25 mmol/L β-mercaptoethanol, 1 mmol/L CaCl₂, pH 7.5). SDS-polyacrylamide gel analysis confirmed that >95% of the plasma membrane glycoprotein Ib had been converted to the water-soluble derivative glyocalcin.

Platelet aggregation. Quinine/quinidine drug-dependent antibodies were assayed by platelet aggregometry as described by Deykin and Hellerstein. Platelet aggregation was followed in PRP stirred at 1,000 rpm at 37 °C. Monoclonal antibody (FMC 23, 100 µg/ml final concentration; FMC 25, 0 to 100 µg/ml final concentration; FMC 25 (Fab₂), 0 to 100 µg/ml final concentration; AN 51, 15 and 150 µg/ml final concentration) was added five minutes before the addition of patient serum (1:10 final) or purified patient IgG (1 mg/ml final concentration). Quinine or quinidine (0.2 mmol/ml final concentration) was added three minutes before patient serum or IgG. Similar experiments were performed with the following platelet stimuli: ristocetin, 1.5 mg/ml; adenosine diphosphate (ADP), 5 µmol/l; collagen, 2 µg/ml; final concentrations. The effect of drug-dependent antibody (purified IgG) was also assessed in an experiment with a patient with severe von Willebrand’s disease (<0.1% of the normal plasma and platelet levels of FVIII/VWF as determined by radioimmun assay). The effect of FMC 25 (Fab₂) (100 µg/ml, final concentration) on aggregation induced by acetone-aggregated IgG (20 µg/ml, final concentration) was performed using washed platelets suspended in Tyrode’s solution. The following experiment was performed to assess whether acetone-aggregated IgG caused the dissociation of platelet-bound FMC 25 (Fab₂). FMC 25 (Fab₂) was labeled by iododecatalyzed radiiodination according to the manufacturer’s recommendations (Pierce). Platelets in Tyrode’s solution (2 x 10⁸ cells/ml, final concentration) were made 5 µg/ml in radiolabeled FMC 25 (Fab₂). To establish equilibrium binding conditions, duplicate 100-µl aliquots were withdrawn at 2, 5, 10, 20, and 30 minutes and overlaid on 1 ml of 20% sucrose in Tyrode’s solution in a Beckman microfuge tube. After centrifugation for two minutes at 8,370 g, the supernatant was carefully aspirated and the pellet counted for radioactivity in a gamma counter. Under the experimental conditions, binding of FMC 25 (Fab₂) was complete within ten minutes and did not alter after this time. To assess the effect of acetone-aggregated IgG, 10 µl of acetone-aggregated IgG (30 µg/ml, final concentration) or Tyrode’s solution was added to 1 ml of platelets in Tyrode’s solution that had been preequilibrated for 30 minutes with 5 µg/ml of labeled FMC 25 (Fab₂). After ten minutes, duplicate 100-µl aliquots were withdrawn and processed as described.

Indirect platelet immunofluorescence. The indirect platelet suspension immunofluorescence test was performed as described by von dem Borne et al., with minor modification. Formaldehyde-fixed platelets were washed twice in EDTA-PBS (0.009 mol/L EDTA, 0.0264 mol/L Na₂HPO₄, 0.14 mol/L NaCl, pH 7.0) and resuspended to a concentration of 4 x 10⁸/ml in EDTA-PBS containing 0.2% (wt/vol) bovine serum albumin. To assess the effect of the mononal antibodies on binding of drug-dependent antibody to platelets, 0.1 ml of purified monoclonal IgG (FMC 25, FMC 25 (Fab₂), FMC 23, and FMC 48: 13 or 67 µg/ml, final concentration) was mixed with 0.1 ml of platelet suspension for 30 minutes at 37 °C. Serum (0.1 ml) and 0.3 ml of 1 mol/ml quinine, 1 mol/ml quinidine or saline were then added and the mixture equilibrated a further 30 minutes at 37 °C. The platelets were then washed three times with EDTA-PBS and mixed with 0.1 ml of optimally diluted fluorescein isothiocyanate (FITC)-tagged monoclonal IgG for 30 minutes at 20 °C. After two more washings with EDTA-PBS, the platelets were resuspended in glycerol-PBS (3:1, vol/vol), mounted on a slide, covered, and examined under the fluorescence microscope. Serum from a patient with an anti-Pl⁺ antibody was used as a positive control. Normal AB serum served as a negative control. Fluorescence was scored between – and + + +. Where normal AB sera was always negative and the immunofluorescence with the anti-Pl⁺ antibody always gave + + + + +. The effect of calcium-dependent proteolysis of platelets on binding of drug-dependent antibody was performed essentially as previously described using normal fixed platelets and platelets fixed after treatment with calcium-dependent protease. To assess whether purified glycoprotein Ib complex, glycoprotein Ib, and glycoprotein IX, competed for binding of drug-dependent antibody, 0.1 ml of purified glycoprotein in 0.01 mol/L Tris, 0.15 mol/L sodium chloride, pH 7.4 (GP Ib complex, 25 µg/ml; GP Ib, 25 µg/ml; GP IX, 10 µg/ml; final concentrations) was mixed with 0.1 ml of serum (patients P and B) and 0.3 ml of 1 mol/ml quinine for 30 minutes at 37 °C. Platelets (0.1 ml) were then added, the mixture equilibrated a further 30 minutes at 37 °C, and then processed as described.

Immunoprecipitation with monoclonal antibodies. Immunoprecipitation using the mouse monoclonal antibodies FMC 25 and AN 51 was performed essentially as previously described. Washed platelets were periodate-labeled as described, except that in the last wash, the EHS buffer was 200 µg/ml in leupeptin. Periodate-labeled platelets were also treated with calcium-dependent protease as described and washed once in the same final buffer. After centrifugation, the labeled platelets were resuspended to 2 x 10⁷/ml in 0.01 mol/L HEPES, 0.15 mol/L sodium chloride, 0.005 mol/L EDTA, pH 7.4. The suspension was made 200 µg/ml in leupeptin and 1 mmol/L in DFP and solubilized by the addition of 11% (vol/vol) Triton X-100 to give 1% (vol/vol) Triton X-100, final concentration. The solubilized platelets were centrifuged at 8,370 g for five minutes in a Beckman microfuge (Beckman Instruments, Spinco Division, Palo Alto, Calif) to remove the insoluble platelet cytoskeletons. The supernatant from platelets that had been treated with human platelet calcium-dependent protease (glyocalcin) and radioiodinated glycoprotein Ib, Ib, and IX were also used in the immunoprecipitation experiments. To 200 µl of the appropriate sample was added 20 µl of a 1:10 dilution of FMC 25 or AN 51 ascites fluid. After two hours at 4 °C, 50 µl of a 1-mg/ml solution of affinity-purified goat antimouse IgG was added and incubated a further 30 minutes at 4 °C. Immunoprecipitation with fixed Staphylococcus aureus cells (Pansorbin) was then performed as previously described.

Immunoprecipitation with drug-dependent antibodies. Immunoprecipitation using the quinidine-dependent antibody (patient D) was performed essentially as previously described. The Triton X-100 lysates of periodate-labeled normal and calcium-dependent protease-treated platelets and the supernatant from platelets that had been treated with human platelet calcium-dependent protease (glyocalcin) were prepared as described. Radioiodinated glycoprotein Ib complex, glycoprotein Ib, Ib, and IX were also used in the immunoprecipitation experiments. To 200 µl of the appropriate sample was added 20 µl of patient serum and 25 µl of saline or quinidine in saline (0.4 mmol/L final concentration) and the mixture left at 4 °C for 16 hours. Immunoprecipitation with fixed S aureus cells (Pansorbin) was then performed as previously described.

Electrophoresis. Samples were prepared for electrophoresis by solubilization in 2% (vol/vol) SDS in the absence (nonreduced) or presence (reduced) of 5% (vol/vol) β-mercaptoethanol. All samples were incubated at 100 °C for five minutes (nonreduced) or ten minutes (reduced) to afford complete solubilization. For one-dimensional analysis, protein was electrophoresed through slab gels according to the method of Laemmli, using a 5% to 20% exponential gradient of acrylamide in the resolving gel and 3% acrylamide in the stacking gel. Protein was stained with Coomassie brilliant blue as
previously described. The tritium distribution in the dried gels was obtained by fluorography according to the method of Bonner and Laskey. For autoradiography, stained gels were dried under vacuum, stored at room temperature next to Cronex 4 medical x-ray film (DuPont Instruments, Wilmington, Del) in cassettes containing intensifying screens, and processed according to manufacturer’s instructions. In some instances, after SDS-polyacrylamide gel electrophoresis of solubilized human platelets, the separated proteins were transferred to nitrocellulose and analyzed by Western blotting. With FMC 25, detection of bound monoclonal antibody used peroxidase-coupled goat antimouse IgG. With drug-dependent antibody, the transferred proteins on nitrocellulose were blocked with normal human serum and probed with 125I-labeled patient IgG (patient D) in the absence and presence of 0.4 mmol/L quinidine.

RESULTS

Characterization of the binding domains on the human platelet glycoprotein Ib complex for FMC 25 and AN 51. The results of immunoprecipitation experiments using periodate-labeled platelets and periodate-labeled platelets that had been exhaustively treated with human platelet calcium-dependent protease are shown in Fig 1. In these experiments, the supernatant from platelets that had been treated with human platelet calcium-dependent protease served as a source of labeled glyocalcin. Both FMC 25 and AN 51 immunoprecipitated the components of the glycoprotein Ib complex (Fig 1, lanes 2 and 3, nonreduced; lanes 9 and 10, reduced, respectively) consistent with our previous report. Similarly, WM 23 directed against an epitope on glycoprotein Ib also immunoprecipitated both glycoprotein Ib and glycoprotein IX (data not shown). The glycoprotein assignments are based on the apparent mol wt under nonreducing and reducing conditions and their correspondence to the platelet glycoprotein profile in the platelet lysate-glycoprotein Ib, nonreduced mol wt = 170,000, reduced mol wt = 145,000 and 25,000, most intensely labeled glycoprotein by the sialic acid-specific periodate labeling procedure; glycoprotein IX, nonreduced and reduced mol wt = 22,000. Two minor bands of slightly lower apparent mol wt than glycoprotein Ib were also evident in the nonreduced gel of the FMC 25 and AN 51 immunoprecipitates (lanes 2 and 3). Although these bands have not been definitively assigned, they may be derived from intramolecular disulfide cleavages in the α-chain of glycoprotein Ib that occur before or during SDS-polyacrylamide gel electrophoresis (cf reference 37). In this regard, bands of similar mol wt could be generated from purified glycoprotein Ib by treatment with low concentrations of β-mercaptoethanol (data not shown). There was also the suggestion of a partially resolved band of slightly higher mol wt than glycoprotein Ib (lanes 2 and 3) and glycoprotein Ibα (lanes 9 and 10). This band may represent glycoprotein Iα, which has been reported to coprecipitate with glycoprotein Ib.

With calcium-dependent protease-treated platelets, FMC 25 immunoprecipitated a trace of unhydrolyzed glycoprotein Ib, a band of mol wt ≈55,000 and glycoprotein IX (lane 4, nonreduced). When examined under reducing conditions, the predominant labeled bands corresponded in mol wt to the β-subunit of glycoprotein Ib and glycoprotein IX (lane 11). The result suggests that after hydrolytic removal of glyocalcin from the α-subunit of glycoprotein Ib, a nonglycosylated remnant of the α-subunit of ≈30,000 mol wt remains membrane-associated and disulfide-linked to the β-subunit of glycoprotein Ib. Although the immunoprecipitates were derived from the same number of platelets, FMC 25 precipi-

![Fluorography of an exponential 5% to 20% SDS-polyacrylamide gel run under nonreduced (lanes 1 through 7) and reduced conditions (lanes 8 through 14) of the FMC 25 and AN 51 immunoprecipitates derived from either the Triton X-100 lysate of periodate-labeled platelets, the Triton X-100 lysate of periodate-labeled platelets that had been treated with human platelet calcium-dependent protease, or the supernatant of periodate-labeled platelets that had been treated with human platelet calcium-dependent protease. Lanes 1 and 8, Triton X-100 lysate of periodate-labeled platelets; lanes 2 and 9, FMC 25 immunoprecipitate derived from the Triton X-100 lysate of periodate-labeled platelets; lanes 3 and 10, AN 51 immunoprecipitate derived from the Triton X-100 lysate of periodate-labeled platelets; lanes 4 and 11, FMC 25 immunoprecipitate derived from the Triton X-100 lysate of calcium-dependent protease-treated platelets; lanes 5 and 12, AN 51 immunoprecipitate derived from the Triton X-100 lysate of calcium-dependent protease-treated platelets; lanes 6 and 13, FMC 25 immunoprecipitate derived from the supernatant of periodate-labeled platelets treated with calcium-dependent protease; lanes 7 and 14, AN 51 immunoprecipitate derived from the supernatant of periodate-labeled platelets treated with calcium-dependent protease. Mol wt markers in order of decreasing mol wt are myosin (200,000), β-galactosidase (130,000), phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000).]
tated more glycoprotein IX and glycoprotein Ib from protease-treated platelets than from control platelets. While the cause of this phenomenon has not been investigated in detail, it presumably represents the increased avidity of FMC 25 for its binding site on the glycoprotein Ib complex after glycocalicin removal. FMC 25 did not immunoprecipitate glycocalicin (lanes 6 and 13). In contrast, AN 51 immunoprecipitated glycocalicin (lanes 7 and 14) and a trace of unhydrolyzed glycoprotein Ib (lanes 5 and 12). AN 51, however, was unreactive with the remnant of the glycoprotein Ib complex that remained membrane-associated (lanes 5 and 12), a result consistent with a preliminary report that AN 51 is directed against an epitope on glycocalicin.39

In addition to the data shown in Fig 1, three lines of evidence were obtained that further defined the binding domain for FMC 25 to be on glycoprotein IX. First, Western blot analysis indicated that FMC 25 was directed against an antigen of 22,000 mol wt, nonreduced and reduced (data not shown). Second, FMC 25 immunoprecipitated purified, radiiodinated glycoprotein IX (Fig 2) but not glycoprotein Ib or glycoprotein Ibα (not shown). Finally, purified glycoprotein IX was tightly bound to a FMC 25-immunoaffinity chromatography column whereas glycoprotein Ibα was not retarded.

Effect of FMC 25 and AN 51 on drug-dependent antibody–platelet interaction. FMC 25 at 100 μg/mL had no effect on platelet aggregation induced by collagen (2 μg/mL) or ADP (5 μmol/L) and little, if any, effect on ristocetin-induced, FVIII/vWF-dependent platelet agglutination (1.5 mg/mL of ristocetin) (data not shown). In contrast, FMC 25 completely blocked quinidine-dependent antibody-induced platelet aggregation at concentrations >1 μg/mL. An effect of FMC 25 IgG on the response of platelets to quinidine-dependent antibody (patient D) could be demonstrated at concentrations as low as 60 ng/mL (Fig 3). FMC 25 (Fab), also blocked quinidine-dependent antibody platelet aggregation at concentrations >1 μg/mL. FMC 23 directed against an antigen on T gondii18 had no effect on drug-dependent antibody–platelet interaction at 100 μg/mL. Similar results were obtained with two other drug-dependent antibodies that could be assessed by platelet aggregometry26 (patient W, quinidine; patient V, quinine). In contrast to FMC 25, which is directed against an epitope on glycoprotein IX, AN 51 directed against an epitope on the glycocalicin region of the α-subunit of glycoprotein Ib had the reciprocal effect on platelet function. AN 51 at 15 μg/mL completely blocked ristocetin-induced, FVIII/vWF-dependent platelet agglutination but was without effect on drug-dependent antibody-induced platelet aggregation at 150 μg/mL (Fig 4). Both FMC 25 and FMC 25 (Fab), at 13 and 67 μg/mL inhibited binding of quinidine-dependent antibody (patient D) to platelets as assessed by indirect platelet immunofluorescence (Table I). AN 51 and the negative controls FMC 23 and FMC 48 (directed against a 24,000-mol wt platelet antigen)17 had no effect on platelet binding of quinidine-dependent antibody. Similar results were obtained with the other drug-dependent antibodies in related experiments (patients W and Y, quinidine; patients B
and P. quinine). There was insufficient serum available from patient V (quinine) for it to be similarly tested.

Experiments were also performed to address the role of platelet Fc receptor and FVIII/vWF in drug-dependent antibody-platelet interaction. FMC 25 (Fab)_2 at 100 μg/mL had no effect on the aggregation of washed platelets by 20 μg/mL of acetone-aggregated IgG, an Fc receptor platelet stimulus, suggesting that the functional response of platelets to drug-dependent antibody is not mediated by way of the platelet Fc receptor. Control experiments (see Materials and Methods) confirmed that under the experimental conditions, the aggregated IgG did not cause the dissociation of platelet-bound FMC 25 (Fab)_2. At equilibrium, 24,500 ± 300 cpm of FMC 25 (Fab)_2 was platelet associated. Ten minutes after the addition of acetone-aggregated IgG (30 μg/mL, final concentration), 24,700 ± 300 cpm of FMC 25 (Fab)_2 remained platelet bound (corrected for dilution factor). Several studies have recently demonstrated that FVIII/vWF is not required for binding of drug-dependent antibody to platelets. However, since the α-subunit of glycoprotein Ib contains the von Willebrand factor binding site (see reference 7 and references therein), it is possible that, although FVIII/vWF may not be required for the initial antibody-platelet binding, FVIII/vWF may be necessary for the subsequent aggregation response of the platelets. However, PRP from a patient with severe von Willebrand’s disease (<0.1% of the normal plasma and platelet levels of FVIII/vWF) gave the same response with quinine and purified patient IgG (patient W) as did normal PRP when assessed by platelet aggregometry. The result is consistent with the lack of effect of AN 51 on drug-dependent antibody-induced platelet aggregation.

Characterization of the binding domains on the human platelet glycoprotein Ib complex for drug-dependent antibody. In similar manner to the analysis used for characterizing the interaction of FMC 25 and AN 51 with the glycoprotein Ib complex, immunoprecipitation with periodate-labeled, control and calcium-dependent protease-treated platelets was used to assess which region of the glycoprotein Ib complex was involved in binding of drug-dependent antibody. Again, the supernatant from platelets that had been treated with human platelet calcium-dependent protease served as a source of labeled glycocalcin.

Table 1. Inhibition of Binding of Drug-Dependent Antibody to Platelets by FMC 25

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Preincubation of platelets with purified murine monoclonal IgG (13 or 67 μg/mL) and indirect platelet immunofluorescence were performed as described in Materials and Methods. Immunofluorescence was scored to + + + +, where AB serum served as a negative control and anti-P61 serum as a positive control. ND, not determined.

Fig 5. Fluorography of an exponential 5% to 20% SDS-polyacrylamide gel run under nonreduced (lanes 1 through 5) and reduced conditions (lanes 6 through 12) of the quinidine-dependent antibody (patient D) immunoprecipitates derived from either the Triton X-100 lysate of periodate-labeled platelets, the Triton X-100 lysate of periodate-labeled platelets that had been treated with human platelet calcium-dependent protease, or the supernatant of periodate-labeled platelets that had been treated with human platelet calcium-dependent protease. Lanes 1 and 6: Triton X-100 lysate of periodate-labeled platelets; lanes 2 and 7: patient serum minus quinidine, immunoprecipitate derived from the Triton X-100 lysate of periodate-labeled platelets; lanes 3 and 8: patient serum plus quinidine, immunoprecipitate derived from the Triton X-100 lysate of periodate-labeled platelets; lanes 4 and 9: patient serum minus quinidine, immunoprecipitate derived from the supernatant of periodate-labeled platelets treated with calcium-dependent protease; lane 12: patient serum plus quinidine, immunoprecipitate derived from the supernatant of periodate-labeled platelets treated with calcium-dependent protease. Mol wt markers are as in Fig 1.
in the FMC 25 and AN 51 immunoprecipitates (Fig 1). The level of glycoprotein Ib detected with patient serum in the absence of drug (lanes 2 and 7) was similar to that found in control experiments with normal serum in the absence and presence of drug (data not shown). With calcium-dependent protease-treated platelets, the patient serum gave the quinidine-dependent immunoprecipitation of the membrane-associated proteolytic remnant of the glycoprotein Ib complex (lane 5, nonreduced: a band of mol wt \( \approx 55,000 \) and glycoprotein IX; lane 10, reduced: predominant labeled bands corresponding in mol wt to the \( \beta \)-subunit of glycoprotein Ib and glycoprotein IX). The patient serum did not give quinidine-dependent immunoprecipitation of glyocalcin (lane 12). In support of the immunoprecipitation data, both patient D and patient W showed similar drug-dependent antibody binding to normal and calcium-dependent protease-treated platelets as assessed by indirect immunofluorescence (Table 2).

Attempts to further define the site on the glycoprotein Ib complex for binding of drug-dependent antibody were uniformly unsuccessful. While preincubation of drug and drug-dependent antibody with the purified glycoprotein Ib complex inhibited subsequent binding of drug-dependent antibody to platelets, both purified glycoprotein Ib and purified glycoprotein IX were ineffective (Table 3). Although drug-dependent antibody immunoprecipitated purified, radioiodinated glycoprotein Ib complex (data not shown), serum from patient D failed to give quinidine-dependent immunoprecipitation of any component of the glycoprotein Ib complex when radioiodinated glycoprotein Ib\(_n\), glycoprotein I\(_b\)\(_p\), and glycoprotein IX were used as the antigenic source. A negative result was also obtained when radioiodinated patient D IgG was used for Western blotting with SDS-polyacrylamide gel-separated platelet proteins electrophoresed under either nonreducing or reducing conditions.

**DISCUSSION**

There is now considerable evidence that quinine/quinidine drug-dependent antibodies interact with a component of the human platelet glycoprotein Ib complex. Several groups have demonstrated that Bernard-Soulier syndrome platelets fail to react with drug-dependent antibody,\(^1\)\(^4\) suggesting that one or more of the absent membrane glycoproteins in this disorder (GP Ib, GP V, GP IX, and a glycoprotein of mol wt \( 100,000 \)\(^6\)) may be the receptor/antigen that reacts with the drug-dependent antibody. In support of this, Kunicki et al.\(^6\) using wheat germ agglutinin affinity chromatography, isolated two major platelet membrane proteins of mol wt 150,000 and 210,000 (probably GP Ib and its "putative analogue"),\(^6\) which inhibited drug-dependent antibody-induced release of \(^{51}\)Cr from labeled platelets, suggesting that one or both of these glycoproteins may be the membrane component that is recognized by the drug-dependent antibody. Further, an antibody obtained from a multitransfused Bernard-Soulier patient\(^4\) inhibited drug-dependent antibody–platelet interaction.\(^3\) In our study, two monoclonal antibodies, both of the IgG\(_1\) subclass, were used to further define the interaction of quinine- and quinidine-dependent antibodies with the glycoprotein Ib complex: AN 51 directed against an epitope on the glyocalcin region of the \( \alpha \)-subunit of glycoprotein Ib (Fig 1) and FMC 25 directed against an epitope on glycoprotein IX (Fig 2). Purified FMC 25 (Fab)\(_2\) fragments or intact antibody completely blocked the aggregation of platelets induced by quinine- and quinidine-dependent antibodies (Fig 3). It was found that FMC 25 and FMC 25 (Fab)\(_2\) fragments directly inhibited drug-dependent antibody-induced platelet aggregation because they inhibit binding of drug-dependent antibody to platelets (Table 1). The effect of FMC 25 and its (Fab)\(_2\) fragments appeared to be specific for this reaction, since aggregation induced by other platelet stimuli, such as ADP and collagen, was unaffected, as was ristocetin-mediated, FVIII/vWF-dependent platelet agglutination. Further, other monoclonal antibodies that were also of the same murine IgG subclass, AN 51 directed against another domain of the GP Ib complex, FMC 48 directed against a distinct platelet membrane protein,\(^17\) and FMC 23 directed against a nonplatelet protein antigen on \( T \) gondii,\(^18\) were without effect on drug-dependent antibody–platelet interaction as assessed by platelet aggregometry or indirect platelet immunofluorescence or both. The available evidence suggests that FMC 25 is directly blocking a binding site on the glycoprotein Ib complex for drug-dependent antibody and not sterically affecting binding of drug-dependent antibody to an adjacent membrane glycoprotein(s). High-titer drug-dependent antibodies have been shown to immunoprecipitate in a drug-dependent manner the components of the glycoprotein Ib complex (this study, Fig 5). In addition, preincubation of drug and drug-dependent antibody with purified glycoprotein Ib complex

**Table 2. Binding of Drug-Dependent Antibody to Normal and Calcium-Dependent Protease-Treated Platelets**

<table>
<thead>
<tr>
<th></th>
<th>Patient D</th>
<th>Patient W</th>
<th>Patient D</th>
<th>Patient W</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(-Q)</td>
<td>(+Q)</td>
<td>(-Q)</td>
<td>(+Q)</td>
</tr>
<tr>
<td>Protease-treated</td>
<td>(+Q)</td>
<td>(+Q)</td>
<td>(+Q)</td>
<td>(+Q)</td>
</tr>
</tbody>
</table>

The calcium-dependent protease treatment of platelets and indirect platelet immunofluorescence were performed as described in Materials and Methods. Immunofluorescence was scored \(-\) to \(+ + + +\), where AB serum served as a negative control and anti-PI\(^{1A}\) serum as a positive control.

**Table 3. Inhibition of Binding of Drug-Dependent Antibody to Platelets by Purified Glycoprotein Ib Complex**

<table>
<thead>
<tr>
<th>Preincubation with</th>
<th>Patient B</th>
<th>Patient P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>(-Q)</td>
<td>(+Q)</td>
</tr>
<tr>
<td>GP Ib complex</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>GP Ib</td>
<td>(+ + + +)</td>
<td>(+ + + +)</td>
</tr>
<tr>
<td>GP IX</td>
<td>(+ + + +)</td>
<td>(+ + + +)</td>
</tr>
</tbody>
</table>

Drug and drug-dependent antibody were preincubated with saline or purified glycoprotein before the addition of fixed platelets (GP Ib complex and GP Ib = 25 \( \mu g/mL\), GP IX = 10 \( \mu g/mL\); final concentrations). Experimental design and indirect platelet immunofluorescence were as described in Materials and Methods. Immunofluorescence was scored \(-\) to \(+ + + +\), where AB serum served as a negative control and anti-PI\(^{1A}\) serum as a positive control.
inhibits subsequent binding of drug-dependent antibody to platelets (Table 3).

Van Leeuwen et al. recently reported a study of four patients with quinine- and quinidine-dependent drug-dependent antibodies. In contrast to the results given here, most patient sera (3/4) were positive by indirect immunofluorescence against Bernard-Soulier platelets while one quinidine-dependent patient serum was negative. They provided evidence from studies in one patient with a quinidine-dependent platelet antibody that the platelet interaction was likely to be mediated by way of the platelet Fc receptor, since (Fab)_2 fragments of the antibody no longer showed quinidine-dependent binding to platelets. Whether the (Fab)_2 fragments were still functional, however, was not tested. Although there is some evidence that glycoprotein Ib may be associated with the platelet Fc receptor, the majority of evidence strongly suggests that drug-dependent antibody-platelet interaction does not occur by way of the platelet Fc receptor. In this study, FMC 25 (Fab)_2 fragments were found to potently inhibit the interaction of quinine- and quinidine-dependent antibodies with platelets (6/6). In contrast, the FMC 25 (Fab)_2 fragments had no effect on the aggregation of washed platelets by acetone-aggregated IgG, an Fc receptor platelet stimulus. In support of this result, Kunicki et al. found that heat-aggregated IgG did not inhibit lysis of 51Cr-labeled platelets by drug-dependent antibodies. Further, most studies have found that drug-dependent antibodies are unrelated with Bernard-Soulier platelets. Pfüllner et al. have recently demonstrated that Bernard-Soulier platelets have normal levels of platelet Fc receptor expression. Because it is now recognized that some patients with Bernard-Soulier syndrome contain low but detectable levels of the glycoprotein Ib complex, this may provide an explanation, in part, for the discrepancy in results of van Leeuwen and co-workers with respect to the other reports. An alternative explanation is that there are a family of Fc receptors on platelets, one of which is specific for drug-dependent antibody. There appear to be two types of Fc receptor on platelets that can be discriminated on the basis of their interaction with immune complexes. One receptor is associated with glycoprotein Ib and binds keyhole limpet hemocyanin-anti-keyhole limpet hemocyanin immune complexes. The second type of receptor, glycoprotein Ib-unrelated, binds ovalbumin-anti-ovalbumin immune complexes. Both types of receptor bind aggregated IgG. If drug-dependent antibodies, therefore, interact with a platelet Fc receptor, this receptor must have characteristics distinct from the two types of Fc receptor that have been previously characterized.

It is now clear that FVIII/vWF is not required for binding of drug-dependent antibody to platelets. However, because the α-subunit of glycoprotein Ib contains the binding domain for FVIII/vWF (see reference 7 and references therein), and because drug-dependent antibodies also bind to the platelet membrane glycoprotein Ib complex, it remained possible that events subsequent to binding of drug-dependent antibody were FVIII/vWF-dependent. PRP from a normal donor and a patient with severe von Willebrand's disease (0.1% of the normal plasma and platelet levels of FVIII/vWF), however, gave an identical aggregation response with quinidine and purified patient IgG. Further, AN 51 blocked ristocetin-mediated, FVIII/vWF-dependent platelet agglutination but did not inhibit drug-dependent antibody binding to platelets or drug-dependent antibody-induced platelet aggregation. The complement-mediated lysis of platelets by drug-dependent antibody is also unlikely to involve FVIII/vWF, since limited proteolysis of platelets with trypsin or chymotrypsin abolishes FVIII/vWF-dependent platelet agglutination but decreases complement-mediated lysis of platelets by drug-dependent antibody by only ≈20%.

Three lines of evidence suggest that the majority of the α-subunit of glycoprotein Ib is not required for interaction of drug-dependent antibody with platelets. First, AN 51 directed against an epitope on the glycolocalicin portion of the α-subunit of glycoprotein Ib had no effect on quinine- and quinidine-dependent antibody platelet-interaction as assessed by platelet aggregometry and by indirect platelet immunofluorescence (Table 1). It is nevertheless possible that the α-subunit of glycoprotein Ib is sufficiently large to accommodate more than one highly specific antibody. However, pretreating the platelets with calcium-dependent protease that specifically cleaves off most of the α-subunit of glycoprotein Ib (glycoalcalin) did not significantly alter binding of drug-dependent antibody to platelets (Table 2). Finally, a drug-dependent antibody gave quinidine-dependent immunoprecipitation of the membrane-bound proteolytic remnant of the glycoprotein Ib complex after treatment of platelets with calcium-dependent protease but did not immunoprecipitate glycoalcalin (Fig. 5). In this regard, Kunicki et al. reported that glycoalcalin lacked receptor activity for drug-dependent antibody as assessed by complement-mediated platelet lysis.

The minimal effective structure found in this study that still bound drug-dependent antibody was the membrane-associated fragment of the glycoprotein Ib complex after treatment of platelets with calcium-dependent protease. This fragment contained glycoprotein IX and an approximately 30,000-mol wt, poorly glycosylated or nonglycosylated remnant of the α-subunit of glycoprotein Ib disulfide-linked to the β-subunit of glycoprotein Ib. Although FMC 25 is directed against an epitope on glycoprotein IX, the inhibition of drug-dependent antibody-platelet interaction by this monoclonal antibody could also conceivably involve steric inhibition of a drug-dependent antibody binding site on the 30,000-mol wt region of the α-subunit or the β-subunit of glycoprotein Ib or both. Attempts to further define the binding domain for drug-dependent antibody, however, were uniformly unsuccessful. Although preincubation of drug and drug-dependent antibody with the purified glycoprotein Ib complex inhibited subsequent binding of the drug-dependent antibody to platelets, both glycoprotein Ib and glycoprotein IX individually were ineffective. The failure of glycoprotein Ib and glycoprotein IX to effectively block binding of drug-dependent antibody to platelets did not appear to be due to their denaturation during the purification procedure. Purified glycoprotein Ib inhibited the FVIII/vWF-dependent agglutination of platelets and glycoprotein IX could be reassocaited with glycoprotein Ib.
body did not immunoprecipitate isolated GP Ibα, GP Ibβ, or GP IX. A similar result was obtained by Western blot analysis. Because both these experiments used SDS-denatured protein, it is not possible to determine whether the failure to bind drug-dependent antibody was due to an effect of SDS on the binding site per se or to the separation of the α- and β-subunits of glycoprotein Ib and glycoprotein IX. Nevertheless, both possibilities are consistent with a conformational requirement of the binding site for drug-dependent antibodies. Further, the combined results suggest that the binding site for drug-dependent antibody on platelets may involve a domain that is disrupted on dissociation of the glycoprotein Ib complex to glycoprotein Ib and glycoprotein IX. If binding is to an individual subunit of the glycoprotein Ib complex, such as GP IX or the β-subunit of GP Ib, the tertiary structure of the binding site could be dependent on the normal interaction of this subunit with the other components of the glycoprotein Ib complex. Alternatively, the binding site could involve structural domains on more than one subunit. Because the three-dimensional structure of the glycoprotein Ib complex is unknown, it is not currently possible to discriminate between these two possibilities.

The combined evidence suggests that drug-dependent antibody–platelet interaction occurs by way of a FVIII/vWF-independent, Fc receptor-independent mechanism. Further, the data suggest that glycoprotein IX plays an important role in the binding of quinine- and quinidine-dependent antibodies to platelets. Nevertheless, several interesting questions remain unresolved. Is a similar mechanism involved with other drug-dependent antibodies? What is the role of the drug in drug-dependent antibody interaction with platelets? These areas of research remain under current investigation.

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Molecular characterization of quinine/quinidine drug-dependent antibody platelet interaction using monoclonal antibodies

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